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Integron Prevalence and Diversity in Manured Soil[▽]

K. G. Byrne-Bailey,[1]# W. H. Gaze,[1]* L. Zhang,[1] P. Kay,[2]† A. Boxall,[2]§ P. M. Hawkey,[3] and E. M. H. Wellington[1]

Department of Biological Sciences, University of Warwick, Gibbet Hill, Coventry, West Midlands CV4 7AL, United Kingdom[1]; Cranfield Centre for EcoChemistry, Shardlow Hall, Shardlow, Derby, Derbyshire DE72 2GN, United Kingdom[2]; and Department of Immunity and Infection, University of Birmingham, Birmingham B15 2TT, United Kingdom[3]

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The levels of integron abundance and diversity in soil amended with pig slurry were studied. Real-time PCR illustrated a significant increase in class 1 integron prevalence after slurry application, with increased prevalence still evident at 10 months after application. Culture-dependent data revealed 10 genera, including putative human pathogens, carrying class 1 and 2 integrons.

Integrons are genetic elements that integrate or excise mobile cassette genes, including those that confer resistance to a wide range of antibiotics (8). Class 1 and 2 integrons are associated with carriage of antibiotic resistance genes in clinically important bacteria, and there is increasing evidence of environmental reservoirs of bacteria carrying these integron classes (9, 10, 18, 19). There is concern that the use of veterinary antibiotics selects for antibiotic-resistant bacteria which, along with antibiotic residues, enter the wider environment via slurry application. The impact of slurry application on environmental reservoirs of antibiotic-resistant bacteria is an important question. This study aimed to investigate the molecular prevalence of class 1 integrons and the diversity of class 1 and 2 integrons in bacteria isolated from pig slurry and from amended clay soils. The study site had a history of long-term application of slurry from tylosin (TY)-fed pigs combined with experimental application of slurry containing sulfachloropyridazine (SCP) and oxytetracycline (OTC). Slurry from tylosin-fed pigs (100 g/ton of feed) was applied to soil annually before the start of the experiment; subsequently, two annual experimental applications were also undertaken, containing 18.85 mg/liter and 2.58 mg/liter of SCP and OTC, respectively. The slurry was applied to the field at the same rate as normal agricultural practice (45,000 liters/ha). Antibiotics were added to model sorption properties in soil and represented real-world concentrations found in pig slurry (4, 5). Slurry was stored for up to 3 months in a holding tank containing a mixture of new and older slurry. Soil samples were taken at several time points over the 2-year experimental period (6).

Over 500 isolates from time points before and after slurry application were screened by PCR for *intI1* and *intI2* (6). Among these, 14.7% ($n = 78$) were positive for *intI1* and/or *intI2*, 5.0% ($n = 27$) carried *intI1* only compared to 8.5% ($n = 45$) for *intI2* only, and 1.1% ($n = 6$) were positive for both *intI1* and *intI2*. Integron prevalence in isolates was dependent on the selective medium used, with the numbers of isolates carrying *intI1* being significantly higher under TY selection (9.9%) than under OTC (4.8%), SCP (3.8%), or no selection (3.6%) and *intI2* being more prevalent under OTC selection (24.8%) than under TY (5.8%), SCP (5.6%), or no selection (0.0%) (chi-square test for comparisons of two proportions). These data suggest that TY and OTC may select or coselect for class 1 and 2 integrons, respectively, with TY selection most likely to occur in the pig gut or in the slurry holding tank, as it was undetectable in soil cores. Conversely, OTC selection may have occurred in the slurry tank or the soil, where it persists (12). There was no clear trend in integron prevalence in isolates at sample points after antibiotic-amended slurry application in either year of the study (data not shown). The molecular prevalence of *intI1* in soils in year 1 was determined by using SYBR green real-time PCR on triplicate DNA extractions at each time point (UltraClean soil DNA kit). PCRs were performed on an Applied Biosystems 7500 Fast system, with the reaction mixtures containing 20 μl 2× power SYBR green PCR master mix (Applied Biosystems), 4 μl primer pairs, 0.4 μl bovine serum albumin (10 mg ml^{-1}), 4 μl template DNA diluted 1:10, and 11.6 μl DNA-free H_2O. The final concentration of each primer was 0.9 μM for 16S (16), int1f2 (TCGTGCGTCGCC ATACA), and int1r2 (GCTTGTTCTACGGCCGTTTGA). Standard curves for absolute quantification were produced from seeded soil inoculated with serial dilutions of *Escherichia coli* SK4903 (IncPβ R751 carrying *intI1* and *qacE*) (17). Molecular prevalence was calculated by dividing target gene abundance by 16S rRNA abundance and multiplying by 100. Corrections were made for 16S rRNA and IncPβ R751 copy number (1, 21). Melting curves were checked for specificity of PCR amplification, and template dilution experiments were carried out to check for PCR inhibition.

The molecular prevalence of *intI1* was significantly lower in preapplication samples than at days 1, 21, 90, and 289 postapplication, using a chi-square test for comparisons of two proportions ($P < 0.0001$) (Fig. 1). In the pig slurry spread onto the trial plots, 0.21% of bacteria carried *intI1* (unpublished data).

* Corresponding author. Mailing address: Department of Biological Sciences, University of Warwick, Gibbet Hill, Coventry, West Midlands CV4 7AL, United Kingdom. Phone: 44 2476522482. Fax: 44 2476523568. E-mail: W.H.Gaze@warwick.ac.uk.

Present address: Department of Plant and Microbial Biology, University of California at Berkeley, Berkeley, CA 94720.

† Present address: School of Geography, University of Leeds, Leeds LS2 9JT, United Kingdom.

§ Present address: Environment Department, University of York, Heslington, York Y010500, United Kingdom.

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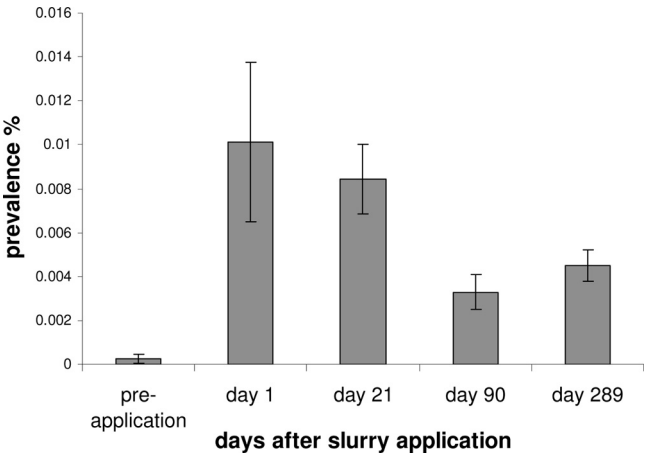

FIG. 1. Molecular prevalence of *intI1* in soil amended with pig slurry. Error bars represent standard errors of the results for three replicate samples (four in preapplication soil); the prevalences are statistically significantly different from one another at all time points (chi-square test, $P < 0.0001$).

In preapplication soils, the *intI1* prevalence was 0.0002%, which was similar to its prevalence in other unpolluted soils tested (unpublished data); this increased dramatically to 0.01% immediately after application and then decreased slightly to 0.008% at day 21 and then to approximately 0.003 and 0.004% at days 90 and 289, respectively. *intI1* prevalence at later time points was still significantly higher than in preapplication soils, indicating that the impact of slurry application on class 1 integron prevalence was still evident after nearly 10 months. This concurs with evidence of *sul1* abundance in manured soils measured using real-time PCR, where a manure effect on abundance was still evident at 61 days postapplication (11). The discrepancy between preapplication soil (which had a history of the addition of slurry from tylosin-fed pigs) and postapplication soil (slurry also containing SCP and OTC) may be due to additional selective pressure exerted by SCP and OTC. In the present study, *Enterobacteriaceae* spp. carrying *intI1* were present in soil leachate samples 164 days after slurry application, again suggesting that integron-positive bacteria, likely to have come from slurry, survived in soil for a considerable length of time.

16S rRNA PCR and sequencing (6) revealed that the *intI*-positive isolates belonged to 10 genera/families (Table 1). The largest number of integrase-positive isolates were *Pseudomonas* spp., which were the only integron-positive genera present throughout the year, including in preapplication samples. Gram-positive *Bacillus* spp. and *Arthrobacter* spp. were also identified, carrying both *intI1* and *intI2* in pig slurry and in soil leachate at day 164 and day 289 postapplication. *Arthrobacter* and *pseudomonas* spp. have previously been isolated from pigsties (2). *Aerococcus viridians* was only isolated from pig slurry; this species is a pathogen of pigs and humans (15). *Psychrobacter* spp. were isolated from pig slurry, preapplication soil, and at day 1 postapplication; members of this genus are also opportunistic pathogens of animals and humans. *Acinetobacter* spp., including *A. lwoffi*, carrying combinations of the two integrase genes were repeatedly characterized in pig slurry and amended soil at day 1 and 21 but were not isolated at later time points or from preapplication cores; this species is an opportunistic human pathogen that is also found as a commensal in healthy individuals (13). *Enterobacteriaceae* spp. were isolated at year 1, day 1, and in soil leachate at day 164 (year 1), and *Enterococcus* spp. were isolated in soil leachate at day 164 only. The majority of integron-bearing genera were isolated postapplication, suggesting that they were introduced via slurry application or were already present in the soil and were selected for by antibiotics contained in applied slurry or resulted from horizontal gene transfer between introduced and indigenous bacteria after slurry application. This correlates with the 50-fold increase in *intI1* observed immediately after slurry application. It is clear that some integron-positive genera, including *Acinetobacter* spp., were only isolated up to day

TABLE 1. Summary of *intI* prevalence and sample identification for each species isolated

Genus as identified by 16S rRNA sequencing	No. (%) of *intI*-positive isolates	*intI* genotype(s)	No. of isolates	Source(s)[a]	Day of isolation postapplication
Acinetobacter	21 (26.9)	*intI1*	3	AS	1, 21
		intI2	16	PS	21
Aerococcus	2 (2.6)	*intI1*	1	PS	
		intI2	1	PS	
Arthrobacter	2 (2.6)	*intI1*	2	AS	289
Bacillus	7 (9.0)	*intI1*	2	SL, AS	164, 289
		intI2	5	PS, SL	164
		intI1, *intI2*	1	PS, SL	164
Enterococcus	1 (1.3)	*intI1*, *intI2*	1	SL	164
Pseudomonas	34 (43.6)	*intI1*	19	P, AS	1, 21, 90
		intI2	14	P, AS	1, 21, 90, 240
		intI1, *intI2*	2	AS	21
Psychrobacter	6 (7.7)	*intI2*	5	PS, P, AS	1
		intI1, *intI2*	1	PS	
Enterobacteriaceae	2 (2.6)	*intI2*	1	AS	1
		intI1, *intI2*	1	SL	164
Stenotrophomonas	1 (1.3)	*intI2*	1	AS	21
Streptomyces	1 (1.3)	*intI2*	1	AS	1
Unknown	1 (1.3)	*intI2*	1	AS	21

[a] PS, pig slurry; P, preapplication soil; SL, soil leachate; AS, amended soil.

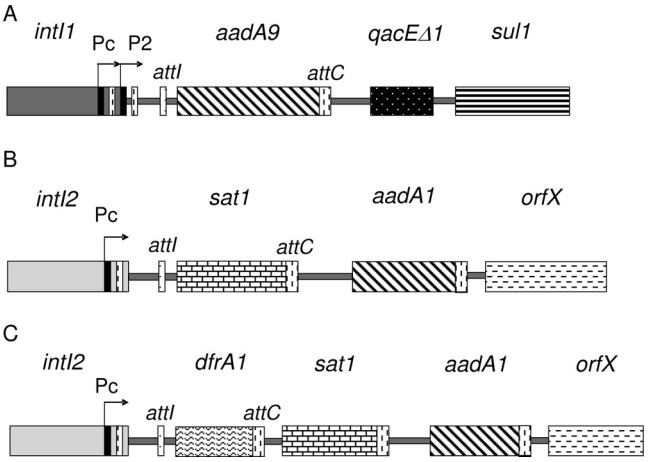

FIG. 2. (A) Schematic of the class 1 integron fragment sequenced from *Arthrobacter arilaitensis* (isolate C361). (B) Diagrammatic representation of class 2 integron PCR fragments, approximately 1,500 bp in length, sequenced from a number of *intI2*-positive isolates. (C) Another class 2 integron structure, similar to that shown in panel B but approximately 2,500 bp in length and with the additional insertion of a *dfrA1* gene cassette.

21, which correlated with a decrease in the molecular prevalence of *intI1* after this time point.

Only six *intI1*-positive isolates contained amplifiable variable regions (18), which contained *aadA* genes (streptomycin/spectinomycin resistance) (GenBank accession number FJ457611); they included *Acinetobacter*, *Aerococcus*, *Pseudomonas*, and *Enterobacteriaceae* spp. and *Arthrobacter arilaitensis*, which also carried an *intI1Δ1* gene and a 3′ conserved segment (3′-CS) that included *qacEΔ1* and *sulI* (Fig. 2A) (7). The latter class 1 integron variable region had 99% similarity at the nucleotide level to a class 1 integron located on the pTet3 plasmid from *Corynebacterium glutamicum* (20). *aadA* genes were isolated from pig manure in a previous study that demonstrated strong selective pressure for streptomycin/spectinomycin resistance in pig farming (3). In conjugal transfer experiments, conducted as described by Byrne-Bailey et al. (6), the *intI1* and *sul1* genes from *Arthrobacter arilaitensis* transferred into *E. coli* K-12 CV601 at a frequency of 3.71×10^{-3} (transconjugants per number of donor cells) and into *Pseudomonas putida* UWC1 at a frequency of 2.98×10^{-3} (transconjugants per number of donor cells), indicating the ability of the mobile genetic element bearing the integron to transfer from a Gram-positive host into Gram-negative recipients.

The variable regions between *intI2* and *orfX* were amplified in *intI2*-positive isolates using primers described by White et al. (22); 4 class 2 integron types were characterized, 10 failed to amplify, and 8 gave a 550-bp sequence containing an *intI2* gene. The third class 2 integron type, which gave a 1,560-bp product and was found in six isolates, was Tn*7* derived, containing *intI2*, a *sat1* gene cassette for streptothricin resistance, an *aadA1* gene cassette, and *orfX* (Fig. 2B) (GenBank accession number FJ469574). The largest of the four class 2 integron types was found in 28 isolates, representing indigenous and introduced bacteria including *Acinetobacter*, *Enterococcus*, *Pseudomonas*, *Psychrobacter*, *Enterobacteriaceae*, *Stenotrophomonas*, and *Streptomyces* spp. and uncultured bacterium EBSCPSA-6117, and gave a 2,300-bp sequence containing a trimethoprim resistance gene (*dfrA1*), streptothricin resistance (*sat1*), streptomycin resistance (*aadA1*), and *orfX* (Fig. 2C) (GenBank accession number FJ492781), an arrangement previously described from *E. coli* isolated from pig feces (14).

Isolates were tested for resistance against eight antibiotics (6). Isolates bearing class 1 integrons demonstrated resistance to more antibiotics than those carrying class 2 integrons (4.4 as opposed to 3.3, respectively; $P = 0.037$, analysis of variance). One of the isolates resistant to all eight antibiotics, C506, identified as an *Enterobacteriaceae* sp., was isolated from soil leachate 164 days after slurry application, demonstrating transport of antibiotic-resistant bacteria of agricultural origin to water catchments.

This study demonstrates that pig slurry-amended soil represents a reservoir of diverse bacterial species carrying class 1 and 2 integrons, with indigenous and introduced bacteria carrying the same integron types. The real-time PCR results demonstrated a significant increase in class 1 integrase prevalence after slurry application, and a significant effect was still observable at day 289 postapplication. The risk of resistance gene transfer from the agricultural environment to the clinic is a matter of controversy. However, it is an accepted fact that farm animals and manure are a source of food- and water-borne human pathogens. It is clear that the same transfer routes will bring the human population into contact with commensal and pathogenic bacteria carrying antibiotic resistance genes that may be further disseminated within the human bacterial flora.

Nucleotide sequence accession numbers. The DNA sequences described in this study have been deposited in the GenBank database under accession numbers FJ457611, FJ469574, and FJ492781.

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